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13. ABSTRACT (Maximum 200 Words)

Besides family history of cancer and an individual's age, no single etiologic factor can identify women at an increased risk for the disease. Approximately 10% of all cases of breast cancer exhibit a familial pattern of incidence. Efforts to identify the genetic basis of familial breast cancer reached fruition some years ago, when the breast-cancer susceptibility genes, BRCA1 and BRCA2 were identified. However, recent studies have suggested that mutations in these genes are associated with a smaller number (20 to 60%) of hereditary breast cancer families than originally estimated, especially in studies that have been based on population-based family materials. Several groups including ours are searching for additional breast cancer susceptibility genes using whole genome scanning approaches, but the success of many of these approaches depend on the underlying heterogeneity of the remaining cancer susceptibility loci. The failure to date to identify additional breast cancer susceptibility genes associated with a high risk of disease suggests that more than one may exist. We have taken the approach that the next BRCA genes will be those that encode for proteins whose functions are linked to important cell regulatory pathways. We have recently found one such candidate BRCA3 protein, referred to as p84N5.

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INTRODUCTION:

A major challenge to breast cancer researchers has been and continues to be the ability to distinguish genetic alterations that are critical to tumor initiation from those that are ephiphenomena of genetic instability. A certain number of breast cancer cases (~10%) are attributed to inherited mutations in highly penetrant breast cancer susceptibility genes, such as *BRCA1* and *BRCA2* [reviewed in (1)]. However, the majority of the tumors occur in women with little or no family history and the molecular basis of these sporadic breast cancers is still poorly defined. Amplification or over-expression of oncogenes (for example c-*MYC*, *ERBB2*, cyclin D1, *EGFR*, γ-synuclein) and loss of *TP53*, *PTEN* (phosphatase and tensin homolog deleted on chromosome 10), *PTCH* (patch), *MKK4* (MAP kinase kinase 4), *BRCA1*, *BRCA2*, and *HIN*-1 (high in normal 1) have been shown to be present in sporadic disease (1-14). Epigenetic changes such as inactivation of *BRCA1* due to promoter hypermethylation have also been described in portion of breast carcinomas (15-17). We have recently discovered that the protein, referred to as p84N5 is abnormally expressed in the vast majority of breast cancer (Guo et al., Cancer Research, 2005).

1.c *Nuclear Matrix Protein, p84N5*

The *p84N5* gene, located on chromosome 18p11.32, was originally isolated on the basis of its ability to encode a protein that specifically associates with the N-terminal half of $p110^{RB}$ (18). This study demonstrated that p84N5 is a nuclear matrix protein that localizes to subnuclear regions associated with RNA processing and binds preferentially to the functionally active, hypophosphorylated form of $p110^{RB}$. The relevance of this interaction for RB function is not completely understood. The p84N5 protein has a region of structural similarity to the death domains of several well-characterized proteins involved in apoptosis, including tumor necrosis factor receptor 1 (TNFR-1) (19). It is thought that p84N5 functions in an apoptotic-signaling pathway initiated from within the nucleus in response to DNA damage (19-21). In addition, the p84N5 protein in cell lines has a specific subcellular nuclear localization that gives a characteristic punctate staining pattern in cells (18). Furthermore, we have found that p84N5 does not appear to be expressed in normal breast ductal epithelial cells, but is expressed in the majority of breast tumor and tumor cell lines. A survey of the various hereditary cancer syndromes find that at least four are proto-oncogenes (i.e., *RET*, *MET*, c-*KIT*, *CDK4*) (22, 23). In general, activated oncogenes in the germline are usually embryonic lethal, yet these proteins are not. However, a second hit is observed (such as LOH) in the cancer, which leads to two mutant copies and/or trisomy (two mutant and one wild-type allele). We hypothesize that p84N5 may be a proto-oncogene, and when over-expressed or mutated contributes to the development of both sporadic and familial forms of breast cancer.

BODY

Progress report year 2

Task 1 (Months 1-18). To evaluate the expression of p84N5 in clinical breast tumor samples and correlate with predictive factors and clinical outcomes.

We have exceeded the expectations of this first task and continue to evaluate the extent of p85N5 expression in cancer. We published a manuscript in April 2005 (Guo, et al. Cancer Res. 2005, 65:3011-3016) and presented this work at the 96th annual AACR meeting in Anahiem, CA. We also have been selected to present our studies as an oral report at the ERA of HOPE meeting scheduled for June 2005. Furthermore, we have two additional manuscripts in preparation which explore the mechanism of aberrant p84N5 expression in cancer. Overall, our results indicated that p84N5 is not only a marker of breast cancer progression, but may also contribute to other forms of cancer, including ovarian. Since, over-expression of and not mutations in (Task 2) p84N5 were observed in breast tumors, we investigated the mechanisms regulating gene expression. We found that aberrant methylation was not involved in regulating *p84N5* expression, however, we did demonstrate that RelA/p65 might play a pivotal role in

regulating its expression. In summary our results suggest that p84N5 could be a predictive marker of tumor progression and potential therapeutic target for treatment of breast cancer. We have included some of the results from our published and unpublished studies.

Task 2 (Months 1-36). To evaluate *BRCA1* and *BRCA2* mutation negative breast cancer-prone kindreds for germline *p84N5* mutations.

We have also made extensive progress addressing the goals outlined in this objective. Unfortunately, no germline or somatic mutations were found in p84N5 leading us to explore other mechanisms regulating its expression in breast tumors (as out line above and shown below). Specifically, we first screened DNA isolated from blood from affected probands in *BRCA1* and *BRCA2* mutation negative breast cancer-prone kindreds for germline *p84N5* mutations by direct DNA sequencing. We screen 45 women with breast cancer who reported at least one first degree relative with breast or ovarian cancer. We observed no deleterious mutations. We also screened 72 cases of sporadic breast cancer and 30 cases of ovarian cancer, as well as 35 cases of EBV-transformed lymphocytes which, were generated from *BRCA1* and *BRCA2* mutation negative breast cancer-prone kindreds by western blot analysis. We used this approach as a means to identify tumor samples potentially carrying a truncated form of the protein. However, all of the samples tested only expressed wild-type p84N5 protein. Examples of these studies are included below.

Task 1-Progress Report-"Evaluate the expression of p84N5 in clinical breast tumor samples and correlate with predictive factors and clinical outcomes."

Expression of p84N5 is also associated with human ovarian cell proliferation

As indicated above, we completed this task and decided to expand our studies. We have observed that the expression of p84N5 in breast tumors is inversely related to hormone receptor status (Guo et al, 2005). Therefore, we decided to compare p84N5 mRNA expression in 6 reduction mammoplasty specimens, including 3 nulliparous premenopausal and 3 parous remenopausal women. We observed that p84N5 mRNA levels were substantially higher in the nulliparous specimens (data not shown). These results indicate that p84N5 is not only deregulated in breast tumors, but also regulated during normal human breast lobular differentiation and might be modified by some hormones, such as human chorionic gonadotropin (hCG). Therefore, we asked whether p84N5 is might also be aberrantly expressed in other hormone dependent tumor-such as ovarian tumors. As expected, p84N5 was highly expressed in all 30 cases of ovarian epithelial tumors examined. Further, we determined p84N5 expression (p84/beta-actin ratio) in primary human ovarian surface epithelial (HOSE) cell cultures (n=10), SV40 Tag immortal HOSE cell lines (n=10) and ovarian tumor cell lines (n=11) by western blotting analysis. We found that p84N5 expression is significantly elevated in immortal cell lines (average value, 0.51) as compared to primary epithelial cells (average value, 0.125; p=0.00024) and reaches its highest level in cancer cell lines (average value, 2.10; p=0.0022) (**Figure 1a, b**).

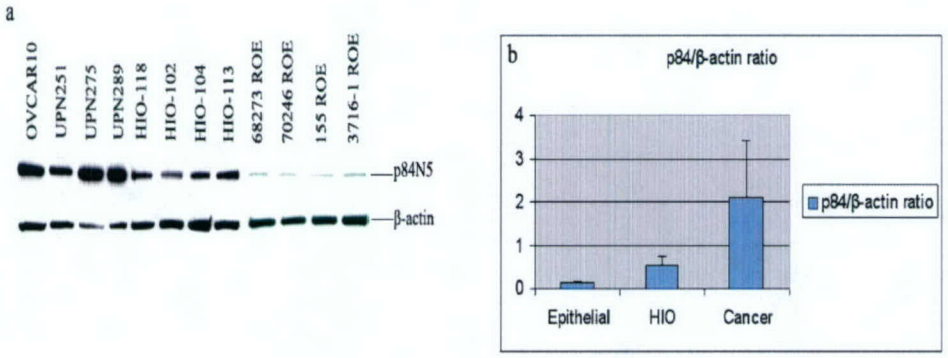
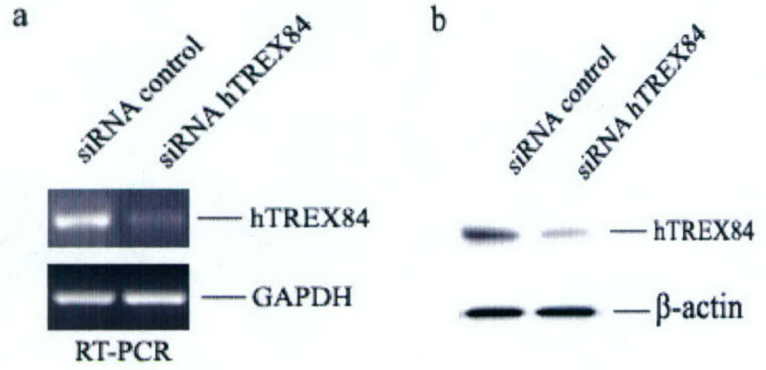


Figure 1. p84N5 is aberrantly expressed in ovarian cancer cells. *(a),* p84N5 protein expression in representative ovarian cancer cell lines (OVCAR10, UPN251, UPN275, UPN289), immortal epithelial cell lines (HIO-118, HIO-102, HIO-104, HIO-113), primary human surface epithelial cells (ROE). Protein samples were separated on a SDS-polyacrylamide gel and proteins were immunoblotted using anti-p84N5 or ß-actin monoclonal antibodies. *(b),* p84N5/ß-actin ratio in primary ovarian epithelial cell cultures (epithelial), immortal epithelial cell lines (HIO) and cancer cell lines (cancer).

To further elucidate the biological significance of the p84N5 in cancer, the siRNA against p84N54 was transfected into an OVCAR 10 cells. RT-PCR analysis using oligonucleotide primers specific to the *p84N5* gene showed that the expression level of the p84N5 transcript decreases 70~80% by following transfection of p84N5 siRNA as compared to cell transfected with control siRNA (**Figure 2a**). p84N5 targeted siRNAs effectively reduced the protein levels of p84N5, but did not affect the levels of non-targeted transcripts such as β-actin (**Figure 2b**). Immunostaining confirmed that p84N5 protein was drastically decreased in majority of the treated cells (**Figure 2c**). Visually, the total numbers of cells decreased significantly following treatment with p84N5-siRNAs as compared to cells treated with transfection reagent or control-siRNA (**Figure 2d**). We observed that cell growth was reduced in cell treated with p84N5-siRNA as compared to control (**Figure 2e**). GuavaNexin assay showed that there were also a reduction of Annexin V-PE and 7-AAD positive cells in cells treated with p84N5-siRNAs as compared to controls and the differences were also not significant ($p>0.05$) (data not shown). In order to look at the mechanism of p84N5 siRNA action, we further determined the cell cycle distribution by flow cytometry and found that the cell numbers in G2-M phase were decreased and cell numbers in G1 phase were increased in OVCAR10 treated with p84N5 siRNA comparing with the cells treated with control siRNA, indicating that p84N5 may be necessary for entry into the G2-M phase (**Figure 2f**). These results indicate that aberrant expression of p84N5 may contribute to ovarian cancer, as well as breast cancer by promoting cell proliferation.



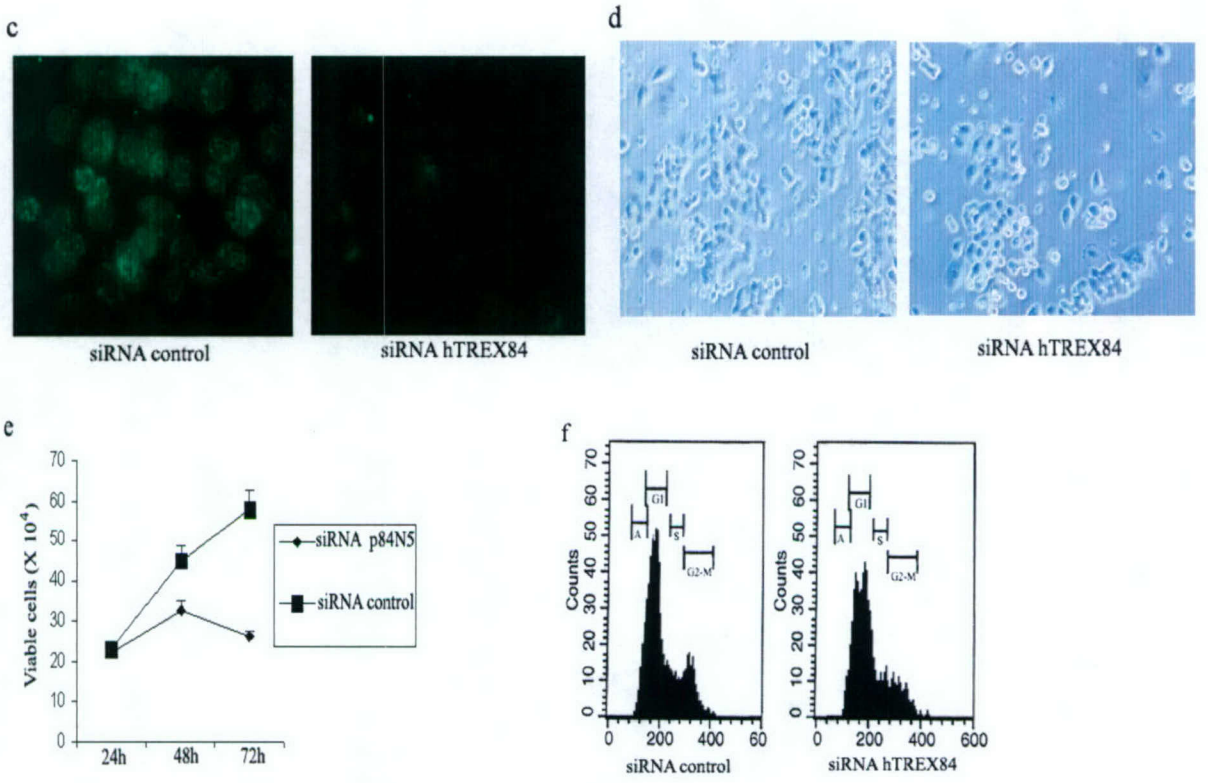


Figure 2. Knock down of p84N5 (hTREX84) leads to defects in cellular proliferation of OVCAR10. (a), Analysis of p84N5 and GAPDH mRNA levels following treatment of cells with siRNA against p84N5 or control siRNA. (b), Analysis of p84N5 and β-actin protein levels treatment of cells with siRNA against p84N5 or control siRNA. (c), Analysis of p84N5 expression following siRNA treatment for 72 hours by immunofluoresence staining in the cells (*left,* cells transfected with control siRNA; *right,* cells treated with TREX84-siRNA). (d), Photomicrographs show the morphology of the following abrogation of p84N5 expression (*left,* tumor cells transfected with control siRNA; *right,* cells treated with p84N5-siRNA). (e), Cell proliferation of tumor cells following abrogation of p84N5. Cell proliferation and apoptosis (data not shown) was examined using Guava ViaCount and Nexin assays, respectively. Plotted is the number of viable cells ($x10^4$) at 24, 48, and 72 hrs after treatment with control siRNA or with p84N5-siRNA. Three independent experiments. (f). FACS analysis of the cells following down-regulation of p84N5 levels. The comparison of cell cycle distribution after 72 hour of treatment with either siRNA (left panel) or p84N5-siRNA (right panel).

p84N5 is a subunit of TREX complex

To gain insight into the biological role of p84N5, we isolated a p84N5-containing multiprotein complex from mammalian cells. This was accomplished by developing a 293-derived stable cell line expressing Flag-tagged p84N5. **Figure 3A** depicts the purification of Flag-p84N5 using anti-Flag antibodies followed by the analysis of the Flag-p84N5 eluate using gel filtration chromatography. This analysis revealed the specific association of p84N5 with polypeptides of 125, 120, 90, 45, 40, and 30K (**Figure 3B and C**). Interestingly, mass spectrometric sequencing of p84N5-associated polypeptides revealed the identity of p84N5 associated proteins as the human counter parts of the yeast TREX complex reported to couple transcriptional elongation and mRNA export (**Figure 3D**). Therefore, we have termed this complex human TREX and p84N5 as hTREX84. Importantly, in contrast to the yeast TREX complex, the human complex was devoid of the RNA export and splicing factors ALY and UAP56. We therefore, asked whether endogenous ALY and hTREX84 form a stable complex which is reflected by

coelution of the two proteins on gel filtration. Analysis of HeLa nuclear extract by Superose 6 sizing fractionation showed distinct chromatographic elution profiles for hTREX84 and ALY proteins indicating that the two proteins are not stably associated (**Figure 3E**). However, consistent with a previous report (24), we observed the association of hTREX and ALY through the UAP56 protein (**Figure 3F**), and that hTREX and ALY colocalize in breast tumor cells as determined by immunofluorescence assays (data not shown). These results indicate that whereas hTREX and ALY may not be stably associated, their interaction is promoted by the UAP56 protein.

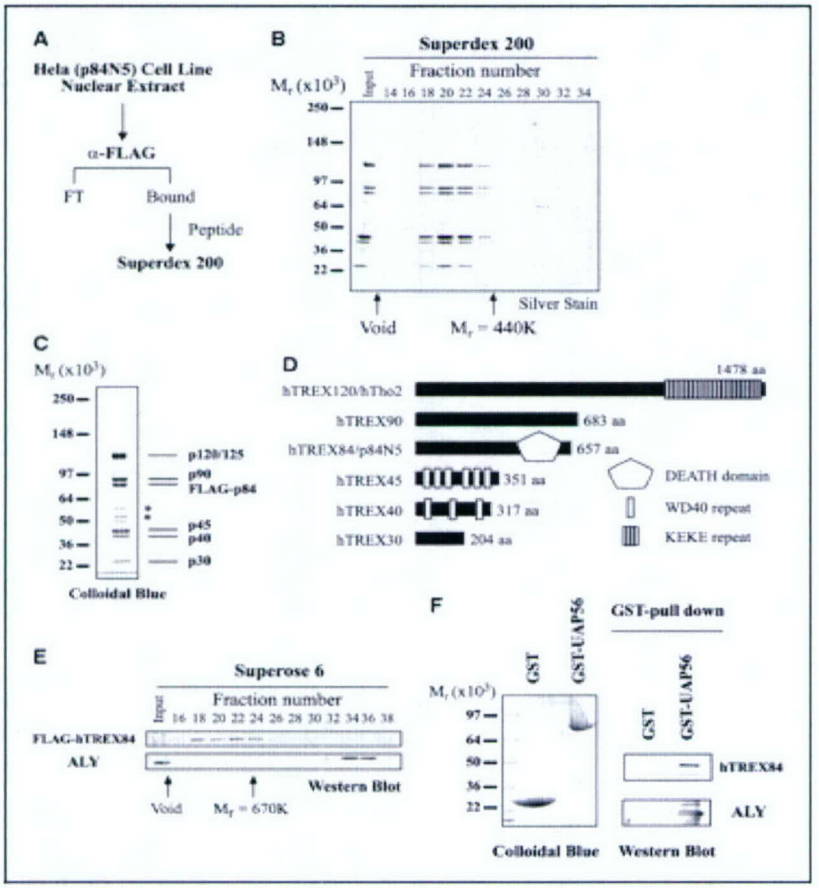


Figure 3. p84N5 is a component of the human TREX complex. *A,* schematic of p84N5 isolation using a 293-derived Flag-tagged cell line. *B,* human TREX complex isolated using the protocol shown in (*A*) was analyzed by silver staining following fractionation on the Superset 200. *C,* colloidal blue analysis of Flag-affinity eluate shown in (*A*). Individual bands were excised and subjected to mass spectrometric sequence analysis. *D,* diagrammatic representation of human TREX subunits. hTREX120, hTREX90, hTREX45, hTREX40, and hTREX30 correspond to Genbank accession nos. AL030996, XM_037945, NM_032361, NM_024339, and BC020599, respectively. *E,* analysis of nuclear extract using Superose 6 gel filtration. Column fractions were analyzed by Western blotting using antibodies (*right*). *D,* GST or GST-UAP56 were used for affinity-purification of human REX and ALY proteins.

Methylation status of p84N5 promoter and exon1 in cancer cells

In our previous report, we found that p84N5 mRNA was high expressed in breast tumors of grade III than those of grade II by quantitative real-time PCR (qPCR). Moreover, p84N5 mRNA was also higher in malignant epithelial cells than normal mammary ductal epithelial cells by qPCR analysis on samples obtained by laser captured micro-dissection (LCM). Therefore, we speculated that deregulation of transcription of p84N5 mRNA may be one of the mechanism of p84N5 protein over-expression in cancer cells. It is well known that methylation of DNA at CpG dinucleotides has been recognized as an

important mechanism for regulation of gene expression in mammalian cells (25, 26). Methylation of cytosines in the CpG sequence located in the promoter region or exon 1 is thought to ensure the silencing of certain tissue-specific genes in non-expressing cells. Aberrant methylation is now considered an important epigenetic alteration occurring in human cancer. Hypermethylation of normally unmethylated tumor suppressor genes correlates with a loss of expression in cancer cell lines and primary tumors. On the other hand, failure to repress genes appropriately by abnormal demethylation of tissue-restricted genes or by hypomethylation of proto-oncogenes could result in the loss of tissue specificity and could promote cancer formation. To elucidate the molecular mechanisms underlying the abnormal transcription of p84N5 in carcinogenesis, a cell line expressing low levels of p84N5 were treated with a demethylating agent 5-aza-2'-deoxycytidine (5-aza-C) at concentrations of 1, 5, 10, 50 μM for 5 days. Total RNAs were isolated and RT-PCR with specific primers to p84N5 cDNA or β-actin cDNA was conducted. The results showed that the intensities of RT-PCR product of p84N5 were increased by the 5-aza-C treatment in a dose dependent manner. By contrast, the products of β-actin were evenly amplified from all the samples, illustrating that the expression of β-actin was not altered by the 5-aza-C treatment (**Figure 4a**). p84N5 protein was also increased in the same manner as assayed by western blotting (**Figure 4b**). Similar results were obtained when using breast tumor cell lines, in which endogenous p84N5 was expressed at low basal levels (data not shown). Genomic DNA was subsequently isolated from these 5-aza-C treated cells and sodium bisulfite DNA sequencing was performed. Surprisingly, the results demonstrated that all the CpG dinucleotides locate on p84N5 promoter and exon1 regions from treated and untreated cells were all demethylated, indicating hypomethylation was likely not the cause for increasing expression of p84N5 mRNA and protein in these cells after 5-aza-C treatment.

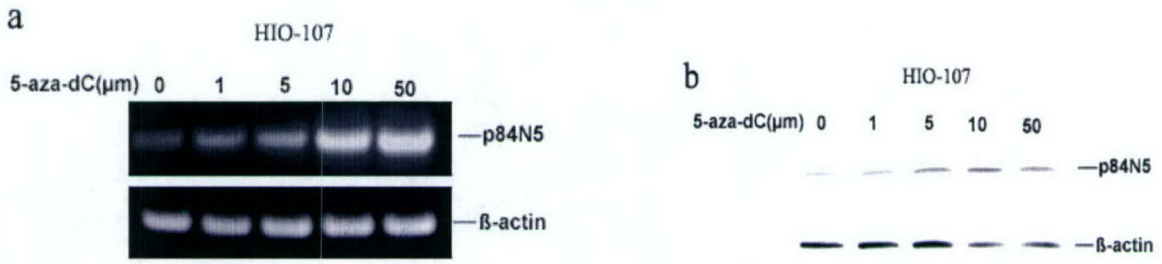


Figure 4. Cells were treated with a demethylating agent 5-Aza-2'-deoxycytidine (5-aza-C) at concentrations of 1, 5, 10, 50 μM, respectively, for 5 days. RT-PCR showed p84N5 mRNA expression (a). Western blot analysis showed p84N5 protein expression (b).

To further rule out a role for methylation in regulating p84N5 expression, we analyzed the promoter and exon 1 of *p84N5* in 8 breast and 7 ovarian cancer cell lines, 10 primary mammary epithelial cell cultures, 20 cases of invasive breast ductal carcinoma, 10 cases of ovarian tumors, as well as their paired normal tissues by sodium bisulfite DNA sequencing. The results showed that the *p84N5* promoter and exon 1 regions in almost all cell lines were unmethylated (**Figure 4a,b**) and that the methylation status did not correlate with p84N5 expression. *p84N5's* promoter and exon 1 regions in most normal tissues were also unmethylated. Occasionally, we observed a few methylated CpG existed in normal tissues (**Figure 4c**). Overall, our results suggested that aberrant methylation of p85N5 is not likely to contribute to its abnormal expression in the majority of breast tumors.

There are several possibilities, which could explain why 5-aza-C can induce p84N5 expression that is independent of promoter methylation. For example, 5-aza-C may have dramatic effects on chromosomes, leading to decondensation of chromatin structure, thus enhancing specific gene expression (27). Another possibility is that 5-aza-C might lead to activation of transcription factors or repression of

inhibitors that regulate p84N5 expression. Although, not yet resolved we have observed that NF-κB is upregulated in many breast tumors and that p84N5 contains consenses NF-κB binding motifs in its promoter (see below).

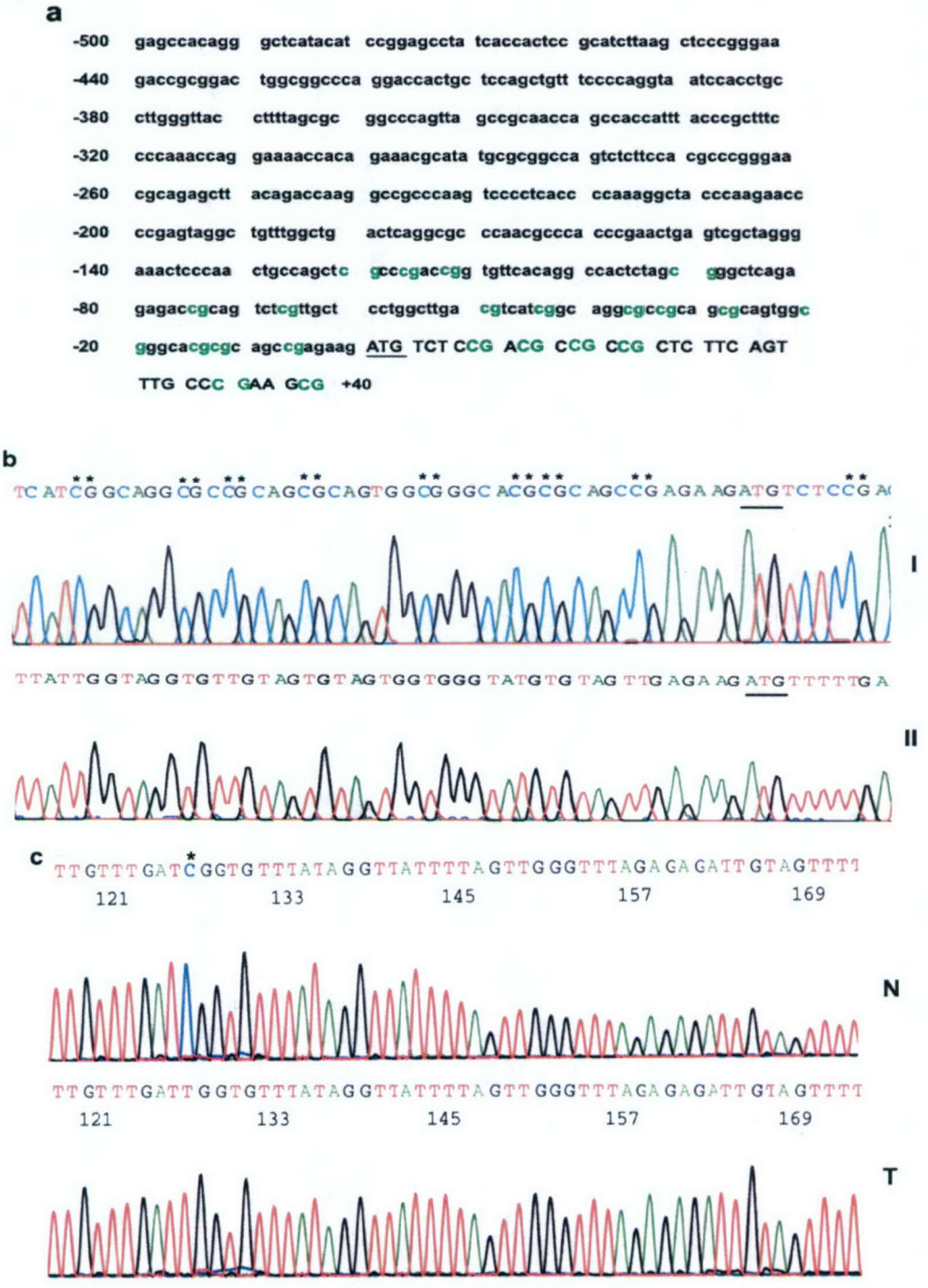


Figure 5. (a). p84N5 promoter and exon 1 region, the nucleotides in green color are CpG sites. Nucleotides are numbered on the right from the ATG translation start codon which is underlined. (b). sodium bisulfite DNA sequencing, from untreated (I) and treated DNA samples (II). Star marker indicates CpG sites. (c). Sodium bisulfite DNA sequencing, from a normal breast tissue (N) and a carcinoma tumor (T). Star maker indicates CpG sites.

NF-κB activation enhances p84N5 expression in immortal and/or cancer cells

To provide a better understanding of the molecular basis of p84N5 over-expression in cell immortalization and carcinogenesis, we took the approach to identify the transcription factor binding sites in *p84N5* promoter (32). The resulting tool AliBaba2 is available at http://wwwiti.cs.uni-magdeburg.de/grabe/alibaba2. 9-SP1, 7-NF1, 4-AP1, 2-NF-κB, together with other transcriptional factors consensus binding sequences were found in the p84N5 promoter by this program. We focus on NFκB and validate it for several clues. Nuclear factor of κB (NFκB) is not a single protein, but a small menagerie of closely related protein dimers that bind a common sequence motif known as the κB site (33). According to Hanahan and Weinberg, tumorigenesis requires six essential alterations to normal cell physiology: self-sufficiency in growth signals; insensitivity to growth inhibition; evasion of apoptosis; immortalization; sustained angiogenesis; and tissue invasion and metastasis (34). NF-κB is able to induce several of these cellular alterations (35), and has been shown to be constitutively activated in some types of cancer cell including breast cancer. Previous studies have documented elevated or constitutive NF-κB DNA-binding activity both in mammary carcinoma cell lines and primary breast cancer cells of human and rodent origin (36-38). This could be correlated with the increased level of epithelial growth factor family receptors (EGFR) (39). Chromatin immunoprecipitation (ChIP) assay is a powerful technique to determine true in vivo binding of transcription factors and other nucleosomal proteins to chromatin (40, 41). We used this assay to determine status of RelA/p65, one subunits of NF-κB, at the promoter of p84N5. After the ChIP protocol, p84N5 gene promoter regions were amplified and analyzed by semiquantitative PCR using specific primer pairs around NF-κB binding regions on the promoter of p84N5 (**Figure 6a**). MDA-MB-231, OVCAR10, OVCAR5 cells cultured for 3 days were subjected to ChIP with and antibody (Ab) to p65. Enrichment of specific DNA sequences in the chromatin immunoprecipitates, which indicates association of p65 to DNA strands within intact chromatin, were visualized by PCR amplification. No binding is seen on immunoprecipitation samples without p65 antibody (**Figure 6b**). These results were further confirmed when we transiently transfected p65 expression plasmid into MCF-10F cells, p84N5 protein was also increased (**Figure 6c**). Moreover, when we knocked down p65 expression by siRNA targeted p65, p84N5 protein also decreased as predicted (**Figure 6d**). In summary, these results show for the first time that RelA/p65 plays a pivotal role in regulating the p84N5 expression.

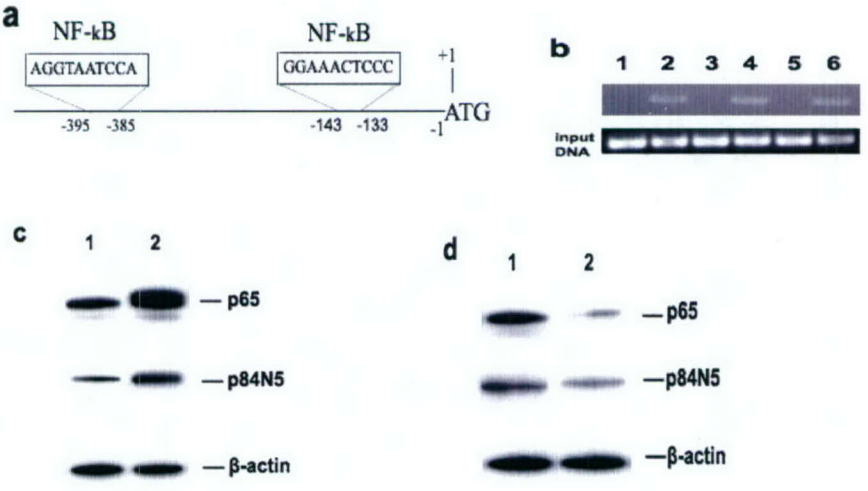


Figure 6. (a) Schematic diagram of the p84N5 promoter, indicating the NF-kB DNA binding motif. (b). ChIP assays of p65 binding to *p84N5* gene promoter in MDA-MB-231 (lane 1, 2); OVCAR5 (lane 3, 4); OVCAR 10 (lane 5, 6). Cells were cultured for 72 h. ChIP assays were then performed with anti-p65 antibody. PCR analysis was performed on immunoprecipitation samples without antibody (lane 1, 3,5), with p65 antibody (lane 2, 4, 6). (c). MCF-10Fcells, transiently transfected with control vector (lane 1), p65 plasmid 48 hours. Western blot analysis for p65, p84N5 and β-actin. (d). MDA-MB-231 cells, western blot analysis of p65, p84N5 and β-actin protein levels treatment of cells with control siRNA (lane 1) and siRNA against p65 (lane 2) for 72 hours.

Additional findings relevant to p84N5's role in the pathogenesis of breast cancer.

In our previous report, we observed that p84N5 protein is undetectable in most normal breast tissues by western blot analysis. However, p84N5 mRNA is detected by RT-PCR analysis. We speculate that a mechanism of p84N5 protein level regulation, which is independent of RNA level regulation, might exist in the normal cell. Since we lack appropriate cultured normal breast epithelial cells for this study, we selected a model 32D cells, which are well-characterized diploid murine hemopoietic cells. 32D cells have an absolute requirement for interleukin-3 (IL-3), and undergo apoptosis when IL-3 is withdrawn. When we induced the cell to differentiate by Granulocytic-Colony Simulating Factor (G-CSF), p84N5 protein rapidly disappear on the second day, however its mRNA decreased but still there even after 5 days. When we treated cells with Lactacystin, a proteasome inhibitor, p84N5 degradation significantly delayed. Further, p84N5 protein was not able to bind ubiqutin, indicating p84N5 protein degradation in the cell differentiation might through ubiqutin independent pathway.

Task 2 Progress Report. To evaluate *BRCA1* and *BRCA2* mutation negative breast cancer-prone kindreds for germline *p84N5* mutations.

We designed four sets of primers specific for p84N5 cDNA and performed DNA sequence analysis (Table 1). The results showed that all cancer cell lines have several nuclear base changes which are different from the original cloned p84N5 cDNA sequence (GenBank NM_005131), but identical to the later predicted p84N5 cDNA sequence (GenBank XM_008756) by automated computational analysis using a gene prediction method, BLAST, supported by mRNA and EST evidence as well as our cDNA sequence (**Table 2**). To further confirm this, we sequenced cDNA from 7 SV40 Tag immortal HOSE cell lines (HIO-102, HIO-113, HIO-114, HIO-117, HIO-121, HIO-135, HIO-166) which were derived and maintained in our laboratory, as well as cDNA from 6 ovarian cancer cell lines (OVCAR2, OVCAR3, OVCAR4, UPN251, UPN275 and A2780). All the ovarian cell lines showed cDNA sequence results identical to those of breast cancer cell lines. Based on these data, we concluded that there are no somatic mutations in p84N5 in these cell lines.

We also tested 45 cases of *BRCA1* and *BRCA2* mutation negative breast cancer-prone kindreds for germline *p84N5* mutations by EMD and direct DNA sequencing. Again, no germline mutations were detected. Interestingly, we also failed to observe polymorphisms in *p84N5* sequence. We also screened 72 cases of sporadic breast cancer and 30 cases of ovarian cancer, as well as 35 cases of EBV-transformed lymphocytes which generated from *BRCA1* and *BRCA2* mutation negative breast cancer-prone kindreds by western blot analysis. No aberrant protein bands were observed (data not shown). Therefore, activation of p84N5 is independent of mutations and is associated with overexpression.

Table 1. Oligonucleotide primer pairs for PCR amplification of the entire cDNA sequence of p84N5

Primer pairs [1]	Oligonucleotide sequences (5'-3')
47U/515L	5'-CTCTTCAGTTTGCCCGAAGC/AAAAGAGCTGAATCCGTCCA-3'
Exons 1-7, fragment length 469 bp	
506U/1033L	5'-CAGTCTTCTGTGGACGGATTC/ATGCCCCTTGAGATATTGGA-3'
Exons 7-12, fragment length 548 bp	
1026U/1576L	5'-CAAGTGAAAAGCTGATGGATTT/TTTAAACTGCTGGTTGGTTGG-3'
Exons 11-19, fragment length 641 bp	
1442U/2083L	5'-GAACAGGCAGACCCTGAAAA/CCAAAACCAGTGGACCTCTT-3'
Exons 18-21, fragment length 642 bp	

[1] Primers were designated by nucleotide position to p84N5 (GenBank XM_00876) corresponding to the 5' position, followed by the letter U for upper (*i.e.,* sense strand) or L for lower (*i.e* ,anti-sense strand).

Table 2. The nuclear base and corresponding amino acid differences of cDNA in the cell lines from the original p84N5 cDNA sequence

Exon	Nucleotide position-database (encoded amide acid)	Nucleotide position-cell lines (encoded amino acid)
6	388G (A)	388T (S)
9	657T	657C
16	1298C (T)	1298T (M)
18	1438C (A)	1438T (V)
19	1458A (M)	1458G (V)
19	1493A (K)	1493G (R)
19	1556A (Q)	1556C (P)

C. KEY RESEARCH ACCOMPLISHMENTS (5/2004 to present):

C.1. "The nuclear death domain protein p84N5; a candidate breast cancer susceptibility gene"

1.a. Demonstrated that p84N5 is expressed at very low levels in normal breast epithelial cells and that it is highly expressed in breast tumors.

1.b. Report that p84 expression correlates with tumor size and the metastatic state of the tumor progression, i.e., identify p84 as a prognostic marker for aggressive forms of human breast cancer.

1.c. Identified that p84N5 is the human counter part of the yeast TREX complex reported to couple transcriptional elongation and mRNA export.

1.d. Demonstrated that p84N5 is over-expressed in both breast and ovarian tumors.

1.e. Abrogation of p84N5 expression leads to growth arrest in both breast and ovarian tumor cell lines.

1.f. Found that 5-aza-C can induce p84N5 expression that is not dependent on p84N5 promoter methylation.

1.g. Demonstrated that RelA/p65 plays a pivotal role in regulating the p84N5 expression.

D. REPORTABLE OUTCOMES (5/2004 to present):

D.1. "The nuclear death domain protein p84N5; a candidate breast cancer susceptibility gene"

1.a. Abstracts

Shan-Chun Guo and A.K. Godwin. Accumulation of p84N5 domain protein is associated with an aggressive phenotype of human breast tumors. Proceedings of American Association of Cancer Research, 44:2421, 2003.

Guo, S., Farber, M.J., Shiekhattar, R. and Godwin, A.K. Over-expression of death domain containing protein-p84N5 in human ovarian cancer cell lines is associated with cell proliferation. Proceedings of American Association of Cancer Research, 44:1805, 2004.

Guo, S., Hakimi, M-A., Baillat, D., Chen, X., Farber, M.J., Klein-Szanto, A.J.P., Cooch, N.S., Shiekhattar, R., Godwin, A.K. Coupling transcriptional elongation and mRNA export to metastatic breast cancers. Proceedings of American Association of Cancer Research, 46:5588, 2005.

Guo, S., Hakimi, M-A., Baillat, D., Chen, X., Farber, M.J., Klein-Szanto, A.J.P., Cooch, N.S., Shiekhattar, R., and Godwin, A.K. Linking transcriptional elongation and mRNA export to metastatic breast cancers. The Era of Hope Breast Cancer Workshop, 2005 (Selected for oral presentation).

1.b. Publications

Guo, S., Hakimi, M-A., Baillat, D., Chen, X., Farber, M.J., Klein-Szanto, A.J.P., Cooch, N.S., Shiekhattar, R., Godwin, A.K. Linking Transcriptional Elongation and mRNA Export to Metastatic Breast Cancers. Cancer Res. 65(8):3011-3016, 2005.

Guo, S., Vanderveer, L., Farber, M.J. Godwin, A.K. hTREX84 is a direct transcriptional target of RelA/p65 in human breast and ovarian carcinomas. In preparation, 2005.

Guo, S., Godwin, A.K. TREX84 served as an early marker in differentiation of myeloid cells. In preparation, 2005.

Book chapters and review articles:

Pan, Z-Z., and Godwin, A.K. Oncogenes, Cancer, and Targeted Therapy. Life and Analytical Science, accepted, 2004.

E. CONCLUSIONS:

E.1. "The nuclear death domain protein p84N5; a candidate breast cancer susceptibility gene"

THO/TREX is a conserved eukaryotic complex containing Tho2, HPR1, MFT1 and Thp2, as well as proteins involved in mRNA metabolism and export such as Aly and UAP56. In the present work, we identified human p84N5 (referred to as hTREX84) as a conserved counterpart of yeast protein HPR1 (Tho1) and for the first time, demonstrated that aberrant hTREX84 expression is associated with not only human breast cancer, but also ovarian cancers. We report that the demethylation agent, 5-aza-2-deoxycytidine, significantly induced p84N5 mRNA and protein expression, suggesting that abnormal expression of p84N5 might be mediated by epigenetic mechanisms. We also have found that RelA/p65 plays a pivotal role in regulating the p84N5 expression. Other mechanisms such as protein abnormal degradation might also contribute p84N5 over-expression in cancer cells. Thus p84N5 might be served as good tumor proliferation maker as well as an ideal target for therapeutic drugs against cancer.

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G. APPENDICES:

Publication:

Guo, S., Hakimi, M-A., Baillat, D., Chen, X., Farber, M.J., Klein-Szanto, A.J.P., Cooch, N.S., Shiekhattar, R., Godwin, A.K. Linking Transcriptional Elongation and mRNA Export to Metastatic Breast Cancers. Cancer Res. 65(8):3011-3016, 2005.

Linking Transcriptional Elongation and Messenger RNA Export to Metastatic Breast Cancers

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Abstract

The biochemical pathways that are disrupted in the genesis of sporadic breast cancers remain unclear. Moreover, the present prognosticating markers used to determine the prognosis of node-negative patient leads to probabilistic results, and the eventual clinical course is far from certain. Here we identified the human TREX complex, a multiprotein complex that links transcription elongation to mRNA transport, as culprit of aggressive human breast cancers. We show that whereas p84N5 (called hTREX84) is expressed at very low levels in normal breast epithelial cells, it is highly expressed in breast tumors. Importantly, hTREX84 expression correlates with tumor size and the metastatic state of the tumor progression. Reduction of hTREX84 levels in breast cancer cell lines by small interfering RNA result in inhibition of cellular proliferation and abrogation of mRNA export. These results not only identify hTREX84 as a prognosticator of breast cancer but also delineate human TREX complex as a target for therapeutic drugs against breast cancer. (Cancer Res 2005; 65(8): 3011-6)

Introduction

Metastatic tumors are the most prevalent cause of death in cancer patients. A major aim in studying metastasis is to understand the mechanism by which cancer cells acquire distinct genetic and epigenetic changes that result in their progression through metastatic states. Recent experiments using microarray studies have expanded our understanding of metastasis in various human tumor samples (1, 2). Although such studies have been powerful for producing gene expression fingerprints of metastatic tumor cells, it has been difficult to assess the contribution of individual genes to the metastasis progression. Breast cancer is the most common malignancy in women and it could be effectively cured if diagnosed at an early stage. The most commonly used predictive molecular markers for breast cancer include Ki-67, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (3). We searched for new prognostic markers that not only could be predictive of the more aggressive forms of breast cancers but also could further provide mechanistic insight into the molecular mechanism underlying metastasis. In this study, we describe the increased expression of TREX84, a subunit of a multiprotein complex involved in transcriptional elongation and mRNA export, in human breast cancer and its intimate association with breast cancer progression and metastasis.

Note: S. Guo and M-A. Hakimi contributed equally to this work.

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Materials and Methods

Primary breast cancer specimens. Human breast tissue specimens used in this study were collected following NIH guidelines and using protocols approved by the Institutional Review Board at Fox Chase Cancer Center. These specimens were surgically obtained from breast cancer patients at Fox Chase from 1991 to 2002. A total 72 primary breast cancer were examined which included 69 invasive ductal carcinomas and 3 invasive lobular carcinomas. Seventy females and two males were included in the study. Ninety percent (65 of 72) of the patients were Caucasian (i.e., white non-Hispanic), 8% (6 of 72) were African American, and 1% (1 of 72) were Asian. The age range was 31 to 97 years with a median age of 56 years. Grading of histologic malignancy of each specimen was assessed according to the system as reported previously (4, 5). Lymphonodal metastatic status was determined by histopathologic examination in each case according to the pTNM classification as proposed by the American Joint Committee on Cancer. Thirty-seven paired normal breast tissues were also obtained from the above patients. All of the samples were snap frozen in liquid nitrogen and kept at −80°C until used. Tissue extracts were prepared as previously described (6).

Affinity purification of Flag-p84. Flag-p84 and a selectable marker for puromycin resistance were cotransfected into HeLa cells. Transfected cells were grown in the presence of 5 mg/mL puromycin, and individual colonies were isolated and analyzed for Flag-p84 expression. To purify the p84 complex, nuclear extract from the Flag-p84 cell line was incubated with anti-Flag M2 affinity gel (Sigma, St. Louis, MO), and after extensive washing with buffer A [20 mmol/L Tris-HCl (pH 7.9), 0.5 mol/L KCl, 10% glycerol, 1 mmol/L EDTA, 2 mmol/L $MgCl_2$, 5 mmol/L DTT, and 0.5% NP40], the affinity column was eluted with buffer A containing Flag peptide (500 mg/mL) according to manufacturer's instructions (Sigma). p84-containing eluate were fractionated on a Superdex 200 (Pharmacia, Peapack, NJ) equilibrated in 0.5 mol/L KCl in buffer A containing 0.1% NP40 and 1 μg/mL aprotinin, leupeptin, and pepstatin. Analysis of nuclear extract on Superose 6 was as described previously (7).

Glutathione *S*-transferase pulldown with UAP56. Control glutathione *S*-transferase (GST, lanes 1) or GST-UAP56 (lane 2) was incubated with HeLa nuclear extract. After washing with BC500 buffer [20 mmol/L Tris-HCl (pH 8), 500 mmol/L KCl, 10% glycerol, 0.2 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride] proteins bound to GST-UAP56 or GST were analyzed by Western blot with p84 antibodies.

Organoid isolation, cell lines, and cell culture. Media and cell culture reagents were prepared by the Cell Culture Facility at Fox Chase Cancer Center. Eighteen cases of organoids were separated and prepared by using collagenase digestion as described previously (8, 9). Six primary cultures of human breast epithelial cells were established and cultured in 199 Medium with 15% fetal bovine serum and insulin (290 units per 500 mL). Six primary cultures of human breast fibroblast cells were cultured in DMEM supplemented with 20% FBS and 1× antibiotic-antimycotic solution. Human breast cancer cell lines MDA-MB-231, MDA-MB-435, MDA-MB-468, MCF-7, BT-20, and ZR-75-1 were cultured in DMEM supplemented with 10% FBS and 1× antibiotic-antimycotic solution. T47D cells were maintained in RPMI supplemented with 10% FBS and 0.2 unit/mL of pork insulin. SKBP-3 cells were maintained in McCoy's 5a medium supplemented with 15% FBS.

Immunofluorescence. Cells grown in monolayer cultures were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100,

and blocked with 10% FCS before antibody staining. Staining by anti-p84 antibodies was visualized with corresponding fluorescein-labeled secondary antibody. All images were acquired with a bio-Rad MRC1000 confocal microscope.

Western blotting assay. After cell lysates were obtained from cell lines or tissues, 30 μg of total protein from each sample were analyzed by Western blotting. Protein extracts were electrophoresed on a 4% to 20% Tris-glycine gel, and the separated proteins were electrophoretically transferred to nitrocellulose for immunodetection. The membrane was blocked in 5% nonfat dry milk in TBST for 1 hour at room temperature and incubated with monoclonal antibody to human p84N5 at a dilution of 1:2000 in TBST + 2.5% nonfat dry milk, followed by horseradish peroxidase-conjugated antimouse secondary antibody (Amersham, Piscataway, NJ) at a dilution of 1:10,000. Immunoblots were reprobed with β-actin monoclonal antibody to confirm equal loading. MDA-MB-435 cell extracts were used as a control sample in each of the experiments. The expression levels of p84 and β-actin detected by immunoblotting were quantitated using the program IMAGE (NIH) for the integrated density of each band. Western blot assays were conducted in duplicate for each sample and the mean value was used for the calculation of protein expression levels.

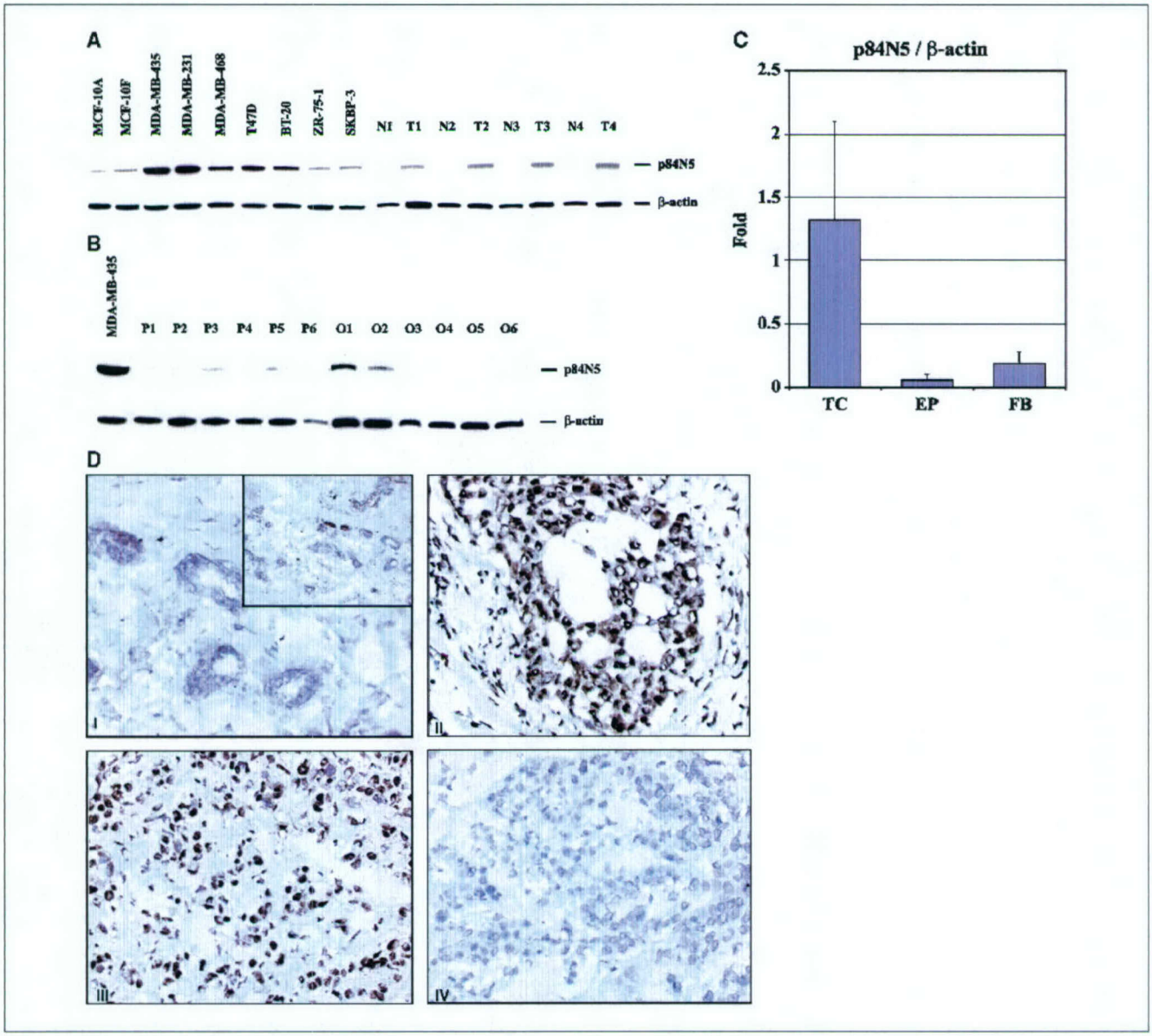

Figure 1. p84N5 is aberrantly expressed in breast cancer. *A,* p84N5 protein expression in immortal breast epithelial cell lines (MCF-10A and MCF-10F), breast tumor cell lines, paired normal (N1-4), and breast cancer (T1-4) tissues. Protein samples were separated on a SDS-polyacrylamide gel and proteins were immunoblotted using anti-p84N5 or β-actin monoclonal antibodies. *B,* p84N5 protein expression in primary breast epithelial cell cultures (P1-P6) and purified organoids (O1-O6) by Western blotting. *C,* p84N5/β-actin ratio in breast cancer cell lines (*TC*), primary breast epithelial cell cultures (*EP*), fibroblast cell cultures (*FB*). *D,* immunohistochemical analysis of frozen sections of normal breast tissue and breast tumor specimens for the p84N5 protein. *I,* p84N5 is weakly expressed in the cytoplasm and nuclei of normal ductal epithelia and lobular epithelia. A few epithelial structures showed moderate immunostain. *Inset,* same region at lower magnification to show overall staining pattern with only few moderately stained ductal structures. *II,* p84N5 is intensively expressed in the cytoplasm and nuclei of a grade 1 invasive ductal carcinoma. *III,* p84N5 is expressed at high levels exclusively in the nuclei of a grade 3 invasive ductal carcinoma. *IV,* previous tumor section evaluated without the primary antibody to serve as a negative control. Magnification 200×.

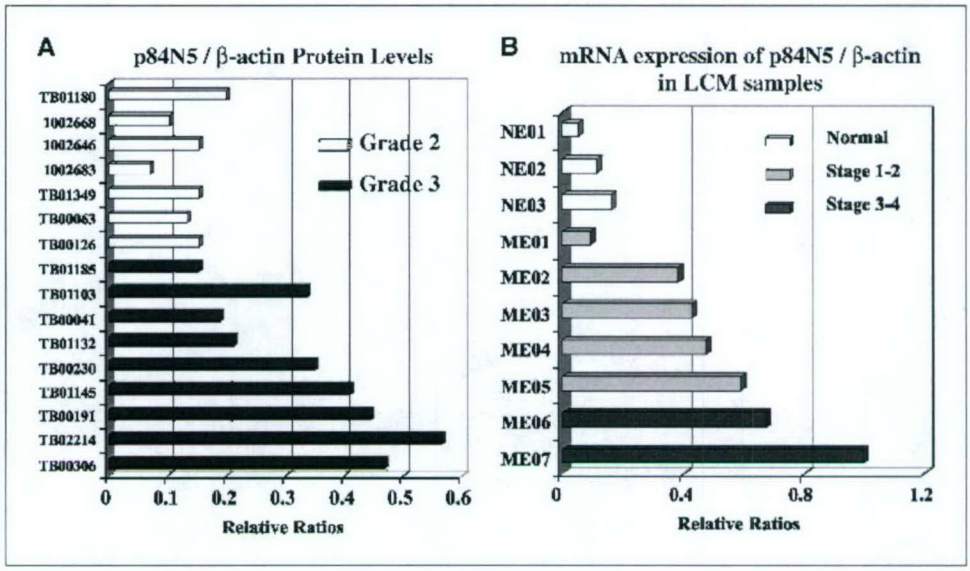

Figure 2. p84N5 displays increased expression in late-stage tumors. *A*, expression of p84N5 by Western blot analysis in the same grade 2 and 3 breast tumors as evaluated. *B*, quantitative real-time PCR analysis of normal mammary lobular epithelial cells (*NE*) and malignant epithelial (*ME*) cells captured by laser capture microdissection. All tumors were grade 3 and were separated based on clinical staging [i.e., combined primary tumor staging (*Tis*), nodal staging (*N0*), and metastatic staging (*M0*)]. ME01-05 were determined to be stage I and II breast tumors, whereas ME06-07 were stage III and IV tumors according to the *AJCC Staging Manual*.

Immunohistochemistry. p84N5 protein immunostaining was carried out with mouse monoclonal p84N5 antibody (Novus Biologicals, Littleton, CO), at a dilution of 1:100. Because the antibody available does not recognize p84N5 in formalin-fixed, frozen sections were used. For frozen section immunohistochemistry, the sections were fixed in cold acetone for 10 minutes and rinsed in cold PBS for 5 minutes. The sections were then incubated in methanol/0.3% hydrogen peroxide for 10 minutes, washed with PBS, and treated with 0.1% Triton X-100 in PBS for 5 minutes and washed with PBS again. The sections were then incubated at 4°C overnight with p84N5 antibody. Reaction products were visualized by immersing the glass slides in 3,3-diaminobenzidine tablet sets (Sigma Fast, Sigma) and counterstained with hematoxylin. A positive control was included in each experiment. As negative controls, either the p84N5 antibody was omitted or sections were washed in 1× PBS.

Laser capture microdissection. Laser capture microdissection (LCM) was done as previously described with minor modification (7). In brief, frozen normal and tumor breast tissue samples were embedded in ornithine carbamyl transferase medium, sectioned in a cryostat at 8-μm thickness, and mounted on nonadhesive glass slides. Fixation was done in 70% ethanol for 60 seconds. Breast epithelial cells were visualized by H&E staining. H&E-stained frozen sections were dehydrated for 30 seconds in 70%, 95%, and 100% ethanol with a final 2-minute dehydration step in xylene. Air-dried sections were then laser captured and microdissected by a PixCell II LCM system (Arcturus Engineering, Mountain View, CA). The normal or malignant mammary epithelial cells to be selectively microdissected away from stroma were identified and targeted through a microscope, and a 15-μm laser beam pulse activated the film on a CapSure LCM Cap (Arcturus Engineering). Approximately 5×10^3 cells were captured for each specimen. Based on careful review of the histologic sections, each microdissection is estimated to contain ~90% of the desired cells. After microdissection, 100 μL of guanidinium isothiocyanate-containing lysis buffer with 0.7 μL mercaptoethanol were applied directly to the microdissected cells adhered on the CapSure LCM cap, samples were placed into a 0.5-mL microfuge tube, and vortexed vigorously. Total RNAs were extracted using the Strata Prep Total RNA Microprep Kit (Stratagene, La Jolla, CA). A DNase treatment was done according to the manufacturer's recommendations. The RNA was resuspended in 20 μL of RNA elution buffer. After being reconcentrated by vacuum without heat, total RNA from each LCM sample was reverse transcribed in a 20-μL reaction as described above.

Quantitative real-time PCR analysis. cDNA mixture (0.63 μL) above was used in a real-time PCR reaction (25 μL total volume) done with Smart Cycle TD (Cepheid, Sunnyvale, CA) following methods recommended by the manufacturer. Optimal conditions were defined as step 1, 95°C for 10 minutes; step 2, 95°C for 15 seconds and 60°C for 60 seconds with Optics, repeated for 50 cycles. The relative mRNA expressions of *p84N5* were adjusted with *ACTB*. The primer and probe sets used for real-time PCR were as follows: *p84N5*, forward primer 5′-GGAACCCTGTGCAATGCTATG-3′ and reverse primer 5′-ACATGTTCTCCTCCTGTTTTCAATT-3′; Taqman probe, (FAM) 5′-ATAAATTAGATGATACTCAGGCCTCAAGAAAAAAGATGGA-3′ (BHQ1). *ACTB*: forward primer 5′-GCCAGGTCATCACCATTGG-3′ and reverse primer 5′-GCGTACAGGTCTTTGCGGAT-3′; Taqman probe, (Cal red) 5′-CGGTTCCGCTGC CCTGAGGC-3′ (BHQ2).

Small interfering RNA transfection and cell proliferation. The small interfering RNA (siRNA) sequences targeting *p84N5* corresponded to the coding region 1652 to 1672 (5′-AATGATGCTCTACTGAAGGAA-3′) relative to

Table 1. Relationship between p84 protein expression and clinicopathologic variables

	n	Mean	Lower bound	Upper bound	*P*
Menopausal status					
Premenopausal	27	0.280	0.165	0.396	0.375
Postmenopausal	45	0.180	0.139	0.260	
Tumor size (cm)					
≤2	21	0.134	0.078	0.191	0.015
>2	50	0.285	0.234	0.363	
Lymph node metastasis					
Negative	31	0.131	0.077	0.185	0.002
Positive	36	0.329	0.232	0.425	
Histologic grade					
2	18	0.143	0.053	0.233	0.033
3	51	0.283	0.213	0.354	
Estrogen receptor					
Negative	23	0.32	0.193	0.448	0.063
Positive	36	0.177	0.122	0.247	
Progesterone receptor					
Negative	28	0.331	0.219	0.442	0.011
Positive	31	0.147	0.092	0.219	

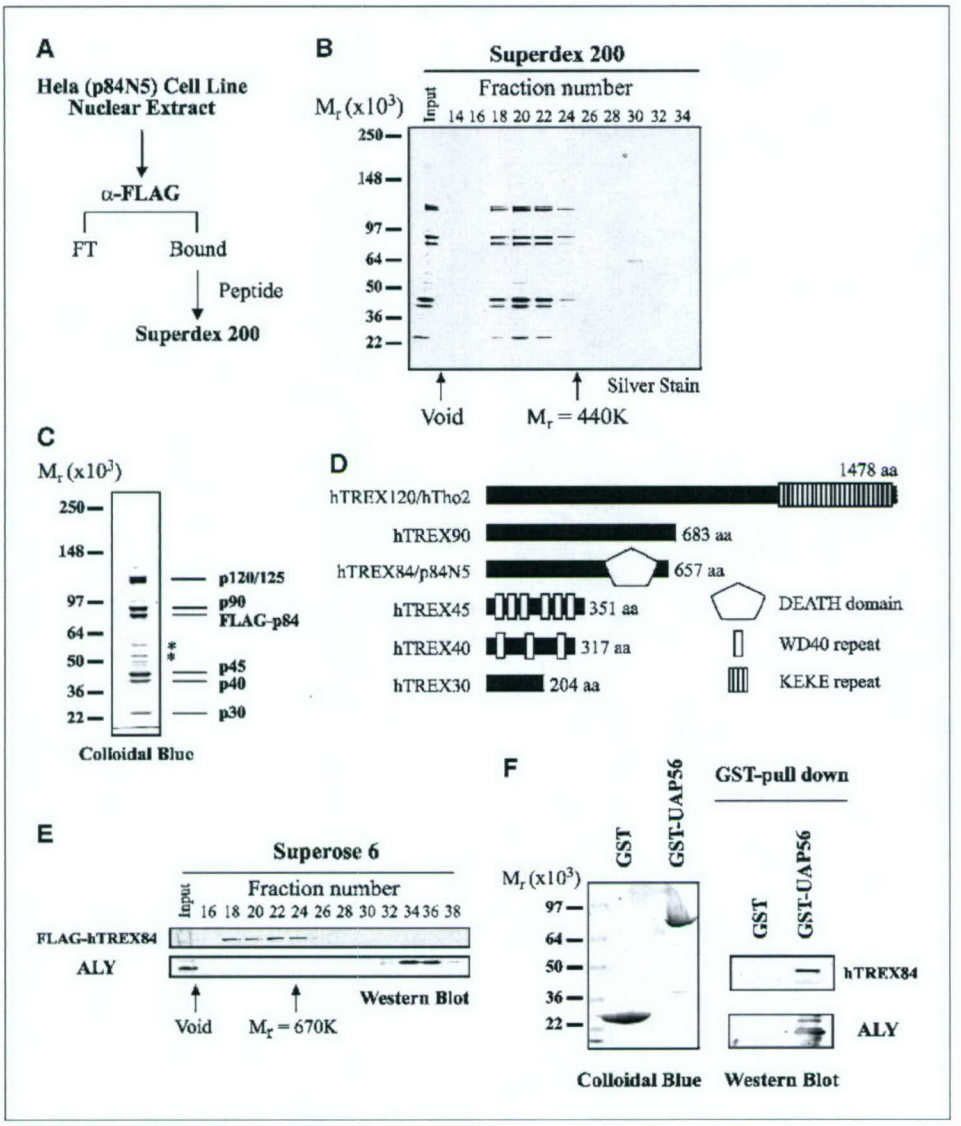

Figure 3. p84N5 is a component of the human TREX complex. *A*, schematic of p84N5 isolation using a 293-derived Flag-tagged cell line. *B*, human TREX complex isolated using the protocol shown in (*A*) was analyzed by silver staining following fractionation on the Superset 200. *C*, colloidal blue analysis of Flag-affinity eluate shown in (*A*). Individual bands were excised and subjected to mass spectrometric sequence analysis. *D*, diagrammatic representation of human TREX subunits. hTREX120, hTREX90, hTREX45, hTREX40, and hTREX30 correspond to Genbank accession nos. AL030996, XM_037945, NM_032361, NM_024339, and BC020599, respectively. *E*, analysis of nuclear extract using Superose 6 gel filtration. Column fractions were analyzed by Western blotting using antibodies (*right*). *D*, GST or GST-UAP56 were used for affinity-purification of human REX and ALY proteins.

the start codon. The corresponding siRNA duplexes with the following sense and antisense sequences were used: 5′-UGAUGCUCUACUGAAGGAAdTdT (sense) and dTdTACUACGAGAUGACUUCCUU-5′ (antisense). A nonspecific control XI siRNA duplex had the following sequences: 5′-AUAGAUAAG-CAAGCCUUACUU (sense) and UUUAUCUAUUCGUUCGGAAUGP-5′ (antisense). All of the siRNA duplexes were synthesized by Dharmacon Research, Inc. (Lafayette, CO) using 2′-ACE protection chemistry.

Cells in the exponential phase of growth were plated at 30% confluence in 6-cm plates, grown for 24 hours, and transfected with siRNA (p84N5 siRNA: 200 nmol/L) using oligofectamine and OPTI-MEM I reduced serum medium (Invitrogen Life Technologies, Inc., Carlsbad, CA), according to the manufacturer's protocol. The concentrations of siRNAs were chosen based on dose-response studies. Silencing was examined 24, 48, and 72 hours after transfection. Control cells were treated with oligofectamine (mock) or transfected using a control siRNA. Cell proliferation and apoptosis was examined using Guava ViaCount and Nexin assays, respectively as previously described (10). All studies were done in triplicates.

Statistical methods. Statistical analyses, including χ^2 and t test, were done using Microsoft Excel software. All statistical tests were two sided, and Ps < 0.05 were considered to be statistically significant. Error bars represent 95% confidence intervals.

Results and Discussion

To identify novel genes whose aberrant regulation may result in sporadic breast cancer, we analyzed the expression profiles of genes in breast tumors using public databases. We focused on p84N5, a nuclear protein containing a DEATH-domain previously reported to associate with *Rb* (11, 12), as one of the genes that displayed increased expression in breast cancers. To directly analyze the expression of p84N5 in breast cancers, we compared the p84N5 protein levels in the breast cancer tissues and the surrounding normal tissues using Western blot analysis. As Fig. 1*A* indicates, whereas cancerous tissues displayed high levels of p84N5 expression, the levels of p84N5 in normal tissues were nearly undetectable (compare N1 through N4 and T1 through T4). Similar increased expression of p84N5 is evident comparing breast cancer cell lines and normal primary epithelial cells or breast organoids (Fig. 1*A* and *B*). We substantiated these results by examining the expression of p84N5 using real-time PCR and immunohistochemistry. Using frozen sections, we detected by immunohistochemistry that normal breast tissue displayed a heterogeneous expression

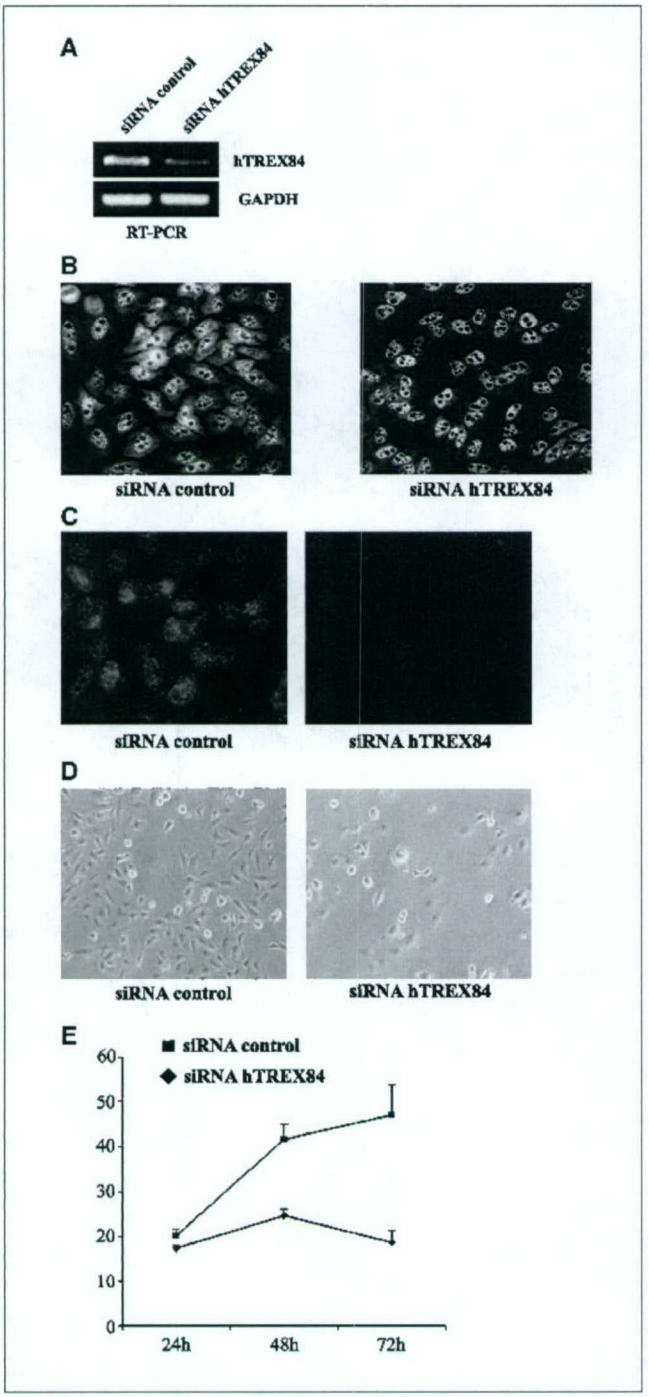

Figure 4. Knock down of TREX84 leads to defects in mRNA export and cellular proliferation. *A*, analysis of TREX84 and GAPDH mRNA levels following treatment of HeLa cells with siRNA against TREX84 or control siRNA. *B*, treatment of HeLa cells with siRNA against TREX84 or control siRNA; treatment of cells with siRNA against TREX84 results in accumulation of mRNA in the nucleus. *C*, analysis of TREX84 expression following siRNA treatment for 72 hours by immunofluoresence staining in MDA-MB-231 tumor cells (*left*, cells transfected with control siRNA; *right*, cells treated with TREX84-siRNA). *D*, photomicrographs show the morphology of the MDA-MB-231 cells following abrogation of TREX84 expression (*left*, tumor cells transfected with control siRNA; *right*, cells treated with TREX84-siRNA). *E*, cell proliferation of breast tumor cells following abrogation of TREX84. Cell proliferation and apoptosis (data not shown) was examined using Guava ViaCount and Nexin assays, respectively. Plotted is the number of viable cells ($\times 10^4$) at 24, 48, and 72 hrs after treatment with control siRNA or with TREX84-siRNA. Three independent experiments.

pattern with a few ductal and lobular epithelial structures exhibiting moderate expression of p84N5, whereas most of the normal breast showed mild or negative expression of the protein (Fig. 1*D*, *I*). Conversely, ductal carcinomas showed an intense and homogeneous expression of p84N5, which is consistent with the Western blot analysis (Fig. 1*C* and *D*, *II-IV*).

We next asked whether p84N5 expression levels were indicative of the aggressive nature of the breast cancers. Comparison of early-stage tumors (grade 2) and those of later stages (grade 3) revealed a marked elevation of p84N5 RNA and protein levels in late-stage tumors (Fig. 2*A* and data not shown). Importantly, analysis of p84N5 levels in a large number of tumors revealed a strong relationship between p84N5 expression levels and lymph node metastasis ($P = 0.002$) and tumor size ($P = 0.015$; Table 1). Other prognostic indicators, including ER positivity ($P = 0.063$) and histologic grade ($P = 0.033$) were also found to be associated with increased p84N5 protein levels. To further confirm these results, lobular epithelial cells from normal breast tissues and malignant epithelial cells from grade 3 tumors were captured by laser capture microdissection and p84N5 levels were analyzed by quantitative real-time PCR (Fig. 2*B*). As Fig. 2*B* attests, the expression levels of p84N5 transcripts are elevated in all but one of the tumors as compared with histologically normal epithelial cells. When these tumors were subdivided based on clinical staging [combined T (tumor size), N (nodal involvement), M (metastatic) classification], p84N5 levels correlated with more aggressive tumors (stage I-II versus III-IV). Taken together, these data indicate that p84N5 is highly expressed in breast cancers and its expression is strongly associated with an aggressive phenotype of human breast tumors.

To gain insight into the biological role of p84N5, we isolated a p84N5-containing multiprotein complex from mammalian cells. This was accomplished by developing a 293-derived stable cell line expressing Flag-tagged p84N5. Figure 3*A* depicts the purification of Flag-p84N5 using anti-Flag antibodies followed by the analysis of the Flag-p84N5 eluate using gel filtration chromatography. This analysis revealed the specific association of p84N5 with polypeptides of 125, 120, 90, 45, 40, and 30K (Fig. 3*B* and *C*). Interestingly, mass spectrometric sequencing of p84N5-associated polypeptides revealed the identity of p84N5 associated proteins as the human counter parts of the yeast TREX complex reported to couple transcriptional elongation and mRNA export (Fig. 3*D*; refs. 13, 14). Therefore, we have termed this complex human TREX and p84N5 as hTREX84. Importantly, in contrast to the yeast TREX complex, the human complex was devoid of the RNA export and splicing factors ALY and UAP56 (13). We therefore asked whether endogenous ALY and hTREX84 form a stable complex which is reflected by coelution of the two proteins on gel filtration. Analysis of HeLa nuclear extract by Superose 6 sizing fractionation showed distinct chromatographic elution profiles for hTREX84 and ALY proteins indicating that the two proteins are not stably associated (Fig. 3*E*). However, consistent with a previous report (13), we observed the association of hTREX and ALY through the UAP56 protein (Fig. 3*F*), and that hTREX and ALY colocalize in breast tumor cells as determined by immunofluorescence assays (data not shown). These results indicate that whereas hTREX and ALY may not be stably associated, their interaction is promoted by the UAP56 protein.

The yeast TREX complex was shown to be intimately involved in the export of mRNA to the cytoplasm (13, 14). We therefore, asked whether human TREX also plays a role in mRNA export.

mRNA was visualized using immunofluorescent analysis using oligo-dt as probes. To address the role of human TREX in mRNA export, hTREX84 protein was depleted using siRNA against hTREX84 following which mRNA levels were analyzed (Fig. 4*A*). Whereas the mRNA in cells treated with control siRNA could be visualized in both the cytoplasmic and the nuclear domains, treatment of cells with siRNA against hTREX84 resulted in the accumulation of mRNA in the nucleus and the loss of cytoplasmic mRNA (Fig. 4*B*). These results indicate that similar to the role for yeast TREX complex, hTREX plays a pivotal function in mRNA export.

Because hTREX84 is highly expressed in aggressive forms of breast cancer, we asked whether reduction of hTREX84 concentrations may slow the proliferative capacity of breast cancer cells. Human breast cancer cell lines express high levels of hTREX84 compared with that of primary breast epithelial cells and organoids (Fig. 1*A* and *B*). To address the proliferative potential of hTREX84, we treated MDA-MB-231 breast cancer cell line with siRNA against hTREX84 (Fig. 4*C*). Treatment of breast cancer cells with siRNA against hTREX84 potently and specifically reduced the proliferative potential of these cells (Fig. 4*D* and *E*). Analyses of these cells using a GuavaNexin assay found no statistically difference for Annexin V-PE and 7-AAD positive cells in siRNA treated cells, indicating the absence of induction of apoptosis (data not shown). Taken together, our finding suggest a role for the hTREX complex in cellular proliferation and following confirmation by other studies conducted among different populations in the future, hTREX84 may serve as a prognostic marker for aggressive forms of human breast cancer. Furthermore, therapeutic interventions that target human TREX should be of tremendous value in the fight against breast cancer.

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